PIK3CA mutation is an oncogenic aberration at advanced stages of oral squamous cell carcinoma

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Phosphatidylinositol 3-kinases (PI3K) are a group of heterodimeric lipid kinases that regulate many cellular processes. Gene amplification and somatic mutations mainly within the helical (exon 9) and kinase (exon 20) domains of *PIK3CA*, which encode the 110-kDa catalytic subunit of PI3K and are mapped to 3q26, have been reported in various human cancers. Herein, 14 human oral squamous cell carcinoma (OSCC) cell lines and 108 primary OSCC tumors were investigated for activating mutations at exons 9 and 20 as well as amplifications in *PIK3CA*. *PIK3CA* missense mutations in exons 9 and 20 were identified in 21.4% (3/14) of OSCC cell lines and 7.4% (8/108) of OSCC tumors by genomic DNA sequencing. An increase in the copy number of *PIK3CA*, although small, was detected in 57.1% (8/14) of OSCC lines and 16.7% (18/108) of OSCC tumors using quantitative real-time PCR. A significant correlation between somatic mutations of *PIK3CA* and disease stage was observed: the frequency of mutations was higher in stage IV (16.1%, 5/31) than in a subset of early stages (stages I–III) (3.9%, 3/77; $P = 0.042$, Fisher's extract test). In contrast, the amplification of *PIK3CA* was observed at a similar frequency among all stages. AKT was highly phosphorylated in OSCC cell lines with *PIK3CA* mutations compared to those without mutations, despite the amplification. The results suggest that somatic mutations of the *PIK3CA* gene are likely to occur late in the development of OSCC, and play a crucial role through the PI3K–AKT signaling pathway in cancer progression. (*Cancer Sci* 2006; 97: 1351–1358)

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is a crucial regulator of many normal cellular processes, such as cell growth, proliferation, motility, survival and apoptosis, and is deregulated in a wide range of human cancers by gain- or loss-of-function of several components of this pathway, including *PTEN*, *AKT* and *PIK3CA*.[1–3] *PIK3CA*, a key element of the PI3K–AKT pathway, is located in chromosomal region 3q26.3, and encodes the 110-kDa catalytic subunit of class IA PI3K. Although an increased DNA copy-number of *PIK3CA* is frequently found in tumors, somatic mutations in *PIK3CA* were recently identified at significant frequencies in various types of human cancer.[4,5] More than 80% of these mutations are clustered in the helical domain encoded by exon 9 and the kinase domain encoded by exon 20. In addition, three hotspot mutations in these exons, E542K, E545K and H1047R, were proven to activate the PI3K–AKT pathway through the phosphorylation of AKT and result in transformation *in vitro*.[6–9] Such evidence demonstrates that oncogenic activation of the PI3K–AKT pathway through the mutated *PIK3CA* is involved in cancer development.

Oral squamous cell carcinoma (OSCC) is a subset of head and neck squamous cell carcinoma (HNSCC) involving the oral cavity, pharynx, and larynx. In Japan, OSCC is relatively common, accounting for more than 5500 deaths in 2003.[10] Oncogenesis is generally considered to involve the progressive accumulation of multiple genetic abnormalities, although little is known about the molecular mechanism behind the development of OSCC. Recently, amplifications and mutations of *PIK3CA* were reported in HNSCC.[11–15] However, no large-scale analysis of *PIK3CA* genetic alterations and their clinicopathological significance has ever been performed in OSCC.

In the present study, therefore, we analyzed 14 human OSCC cell lines as well as 108 primary OSCC tumors with regard to the frequency of mutations within hotspot regions and the changes in the genomic copy-number of *PIK3CA*. Then, we evaluated if there were any statistically significant associations between *PIK3CA* gene status and the clinical characteristics of OSCC patients. Furthermore, to investigate which genetic event, the mutation or amplification of *PIK3CA*, is essential in activation of the PI3K–AKT signaling pathway, we compared the expression of *PIK3CA* and the phosphorylation of AKT in OSCC cell lines in which the *PIK3CA* and *PTEN* gene status was confirmed.

Materials and Methods

Cell lines. The human OSCC cell lines Ca9-22, HSC-2, HSC-3 and HSC-4 were obtained from Japan Health Science Foundation (Osaka, Japan). HOC313, HOC815, HSC-5, HSC-6, HSC-7, NA, OM1, OM2, TSU and ZA, human OSCC cell lines, were generously donated by Dr T. Amagasa (Tokyo Medical and Dental University, Japan). RT7, immortalized human oral keratinocytes, and KYSE70 and TE10, human esophageal squamous cell carcinoma (ESCC) cell lines, were kindly provided by Dr N. Kamata (Hiroshima University Faculty of Dentistry, Japan) and Dr Y. Shimada (Kyoto University Graduate School of Medicine, Japan), respectively. OSCC and ESCC cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium, respectively, supplemented with streptomycin (100 μg/mL), penicillin (100 units/mL), 2 mM glutamine, and 10% fetal bovine serum (FBS). RT7 was maintained in KGM-2 Bullet Kit (Cambrex, Walkersville, MD, USA).

To determine the growth rate of each cultured cell line, 1.0×10^4 cells/well were inoculated in 24-well plates and the cell numbers in triplicate wells were evaluated after 7 days treatment in 0.5% FBS with LY294002 (Cell Signaling Technology, Beverly, MA, USA) by the MTT [3-(4,5-dimethylthiazol-2-yl)-

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Table 1. Primers used for PCR and sequencing for screening of mutations

Gene	Sense	Antisense	Sequencing
PIK3CA			
Exon 9	5′-GATTGGTTCTTTCCTGTCTCTG-3′	5′-CCACAAATATCAATTTACAACCATTG-3′	5′-TTGCTTTTTCTGTAAATCATCTGTG-3′
Exon 20	5′-TGGGGTAAAGGGAATCAAAAG-3′	5′-CCTATGCAATCGGTCTTTGC-3′	5′-TGACATTTGAGCAAAGACCTG-3′
EGFR			
Exon 18	5′-TCAGAGCCTGTGTTTCTACCAA-3′	5′-TGGTCTCACAGGACCACTGATT-3′	5′-TCCAAATGAGCTGGCAAGTG-3′
Exon 19	5′-AAATAATCAGTGTGATTCGTGGAG-3′	5′-GAGGCCAGTGCTGTCTCTAAGG-3′	5′-GTGCATCGCTGGTAACATCC-3′
Exon 21	5′-GCAGCGGGTTACATCTTCTTTC-3′	5′-CAGCTCTGGCTCACACTACCAG-3′	5′-GCTCAGAGCCTGGCATGAA-3′
PTEN			
Exon 1	5′-GCAGCTTCTGCCACTTCTCT-3′	5′-CATCCGTCTACTCCCACGTT-3′	
Exon 2	5′-CTCCAGCTATAGTGGGGAAAA-3′	5′-CTGTATCCCCCTGAAGTCCA-3′	
Exon 3	5′-TGGTGGCTTTTTGTTTGTTT-3′	5′-CATGAATCTGTGCCAACAATG-3′	
Exon 4	5′-AAAGATTCAGGCAATGTTTGTT-3′	5′-TCTCACTCGATAATCTGGATGAC-3′	
Exon 5	5′-GGAATCCAGTGTTTCTTTTAAATACC-3′	5′-TCCAGGAAGAGGAAAGGAAAA-3′	
Exon 6	5′-ATGGCTACGACCCAGTTACC-3′	5′-TTGGCTTCTTTAGCCCAATG-3′	
Exon 7	5′-TGCTTGAGATCAAGATTGCAG-3′	5′-GCCATAAGGCCTTTTCCTTC-3′	
Exon 8–1	5′-GTGCAGATAATGACAAGGAATA-3′	5′-ACACATCACATACATACAAGTC-3′	
Exon 8–2	5′-TTAAATATGTCATTTCATTTCTTTTTC-3′	5′-CTTTGTCTTTATTTGCTTTGT-3′	
Exon 9	5′-TGTTCATCTGCAAAATGGAAT-3′	5′-CAAGTGTCAAAACCCTGTGG-3′	

2,5-diphenyltetrazolium bromide; DMSO] method using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Results were normalized to the cell numbers in control cultures treated with DMSO alone.

Tumor samples. With the approval of local institutional review boards, 50 formalin-fixed paraffin-embedded OSCC samples in Japan and 58 frozen primary OSCC samples in Thailand were selected for study. Clinical and laboratory data on all of the 108 OSCC patients were collected from the patient records. The TNM classification of Union International Contre le Cancer (UICC) was used. Genomic DNA was extracted from the cell lines and the frozen tissue using a Genomic DNA Purification kit (Gentra, Minneapolis, MN) according to the manufacturer's instructions. DEXPAT (TaKaRa BIO, Otsu, Japan) was also used in the extraction of genomic DNA from the formalin-fixed paraffin-embedded samples. Smoking status was categorized as follows: never-smokers, individuals who had smoked fewer than 100 cigarettes in their lifetime; former smokers, those who had quit at least 1 year before interview; current smokers, those who were currently smoking or had stopped smoking within the previous 1 year; and ever-smokers, current and former smokers combined.

Screening for mutations. *PIK3CA*, epidermal growth factor receptor (*EGFR*) and *PTEN* were amplified by polymerase chain reaction (PCR) from genomic DNA (10 ng per sample) with specific oligonucleotide primers. PCR products were sequenced directly with the BigDye terminator method (Applied Biosystems, Foster City, CA, USA) on a capillary autosequencer (ABI Prism 3100) using the sequencing primers. The primers used for PCR and sequencing are shown in Table 1. All samples found to have a mutation were subsequently sequenced in the reverse direction to confirm the mutation using the antisense PCR primers. Then the mutation was further verified by sequencing of a second PCR product derived independently from the original template.

Screening for gene amplification. Gene amplification of *PIK3CA* was assessed by SYBR Green quantitative PCR. In this analysis, we used RT7 as the normal counterpart of OSCC cell lines or six normal genomic DNAs extracted from normal lymphocytes as a control for primary tumors, and KYSE70 and TE10 as a positive control. *COL7A1*, mapped to 3q21, was selected as a control for single copy genes. PCR was carried out with the SYBR Green PCR Master Mix (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System. The primer pairs used were as follows: for *PIK3CA*, sense 5′-ATCTTTTCTCAATGATGCTTGGCT-3′, and antisense 5′-CTAGGGTGTTTCGAATGTATG-3′ for *COL7A1*, sense 5′-ACCCAGTACCGCATCATTGTG-3′, and antisense 5′-TCAGGCTGGAACTTCAGTGTG-3′.

We analyzed all results on a standard curve derived from a known concentration of sample. Six normal samples were also used in each assay, and the mean value was used to normalize the data and correct for interassay variation. The *PIK3CA* gene copy-number was calculated by dividing its value by the *COL7A1* values. A value exceeding fourfold the standard deviation was considered to represent high-level DNA amplification.

Real-time RT-PCR. Real-time reverse transcription-PCR (RT-PCR) was performed using an ABI Prism 7900 Sequence Detection System (Applied Biosystems), the SYBR Green PCR Master Mix (Applied Biosystems), and random-primed cDNAs. The primer pairs used were as follows: for *PIK3CA*, sense 5′-TTAGCTATTCCCACGCAGGA-3′, and antisense 5′-CACAATAGTGTCTGTGACTC-3′ for *GAPDH*, sense 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, and antisense 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. Expression levels of the *PIK3CA* gene were based on the amount of the target message relative to that of the *GAPDH* transcript as a control, to normalize the initial input of total RNA.

Western blot analysis. Cells were washed with phosphate-buffered saline and lyzed in cell lysis buffer (50 mM Tris-HCl pH 7.5, 0.5% NP-40, 150 mM NaCl, 2 mM $NaVO_3$, 100 mM NaF, 10 mM pyrophoric acid, and 1 mM EDTA) then boiled for 3 min. These cell lysates were analyzed by western blotting using anti-AKT, anti-p-AKT (Ser-473), anti-p-AKT (Thr-308), and anti-PTEN rabbit polyclonal antibodies (Cell Signaling Technology), and anti-β-actin monoclonal antibody (Sigma, St Louis, MO, USA).

Statistical analysis. The associations between *PIK3CA* mutations and clinicopathological characteristics were evaluated with Fisher's exact test and the chi-squared test. A *P*-value less than 0.05 was defined as being statistically significant.

Results

Identification of somatic mutations of *PIK3CA* in OSCC. We first investigated the genomic DNA of 14 human OSCC cell lines by direct sequencing of PCR products, and found that HSC-2, -3, and -4 each harbored a different missense mutation in the *PIK3CA* gene, A3140G in exon 20, and A1634G and G1633A in exon 9, corresponding to an amino acid change of H1047R, E545G, and E545K, respectively (Table 2 and Fig. 1A). Using

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Table 2. Summary of *PIK3CA* mutations in oral squamous cell carcinoma

Exon	Nucleotide	Amino acid	Cell lines	Primary cases
9	G1624A	E542K*	0	2
9	G1633A	E545K*	1	1
9	A1634G	E545G	1	0
9	A1637T	Q546L	0	1
20	A3127G	M1043V	0	1
20	A3140G	H1047R*	1	1
20	G3145A	G1049S	0	1
20	G3145C	G1049R	0	1
Samples with mutations			3/14 (21.4%)	8/108 (7.4%)

*A hot spot mutation previously reported to elevate kinase activity compared with the wild type.

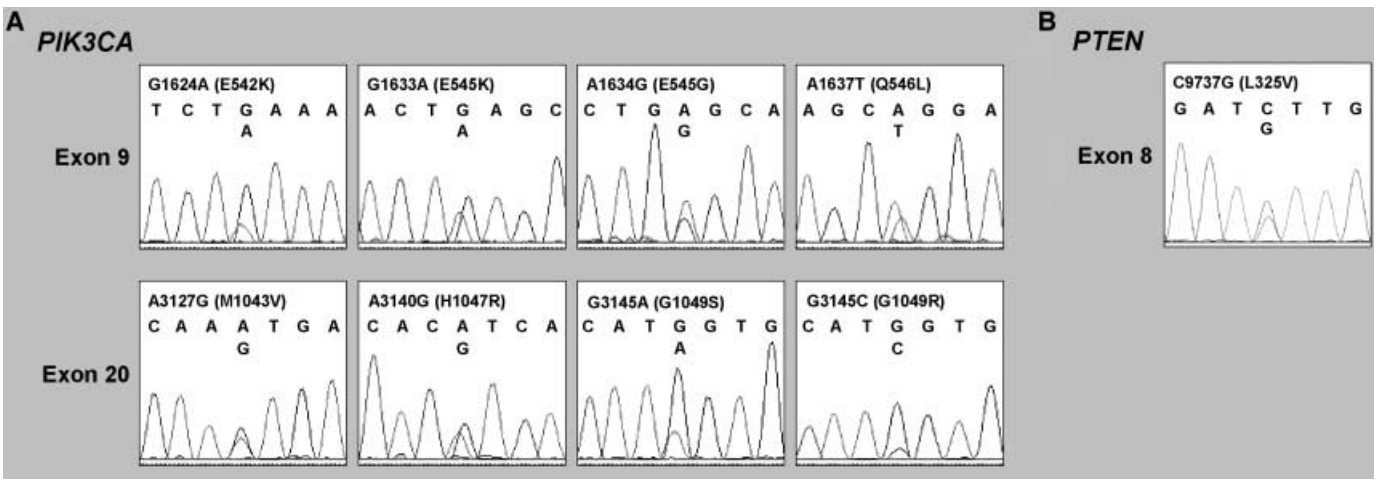

Fig. 1. Sequence chromatograms of missense mutations as determined by automated sequence analysis. (A) Eight different missense mutations in exons 9 and 20 of *PIK3CA*. (B) One missense mutation (C973G) in exon 8 of *PTEN*. These were directly sequenced in two repeat examinations with independent genomic PCR.

the same method, we also confirmed the absence of *PIK3CA* mutations in two ESCC cell lines, KYSE70 and TE10, used as a positive control in the subsequent real-time PCR analysis and western blot analysis.

In order to compare with frequency of mutation of *PIK3CA* in OSCC cell lines, we screened for mutations in exons 18, 19 and 21 of the *EGFR* gene and exons 1–9 of the *PTEN* gene. No mutation or homozygous deletion of *EGFR* was detected in any of these cell lines. However, a missense mutation (C973G) in exon 8 of *PTEN*, corresponding to L325V, was found in HOC815 cells (7.1%, Fig. 1B). This mutation of the *PTEN* gene has not been reported previously.

Then, as the frequency of *PIK3CA* mutations in OSCC cell lines (21.4%) was not lower than in a previous study of various other cancers, we examined 108 OSCC primary tumors for the presence of somatic mutations in exons 9 and 20 of *PIK3CA*. The clinical characteristics and genetic alterations to *PIK3CA* in OSCC patients are summarized in Table 3. In eight specimens, we found missense mutations (7.4%): G1624A, G1633A, and A1637T in exon 9, and A3127G, A3140G, G3145A, and G3145C in exon 20, corresponding to the changes E542K, E545K, Q546L, M1043V, H1047R, G1049S, and G1049R at the amino acid level, respectively (Table 2 and Fig. 1A).

These analyses of OSCC cell lines and primary tumors led us to identify eight independent missense mutations of *PIK3CA*: two of three mutations in cell lines and four of eight mutations in primary tumors were hotspots with confirmed oncogenic transforming activity *in vitro*.[6–9] All of the mutations detected in this study were heterozygous. However, while seven of these missense mutations have already been reported on various types of cancers, there is no information available about A1637T (Q546L). As A1637T in exon 9 of *PIK3CA* and C973G in exon 8 of *PTEN* are not stored in the COSMIC (Catalog Of Somatic Mutations In Cancer), a database of the Sanger Institute, or the NCBI SNP database (gene ID: 5290 and 5728, respectively), this is the first report of these missense mutations. Four single nucleotide polymorphisms C3075T (T1025T) in exon 20 of *PIK3CA* registered in the NCBI SNP database were also observed heterozygously in primary tumors.

Identification of *PIK3CA* amplification in OSCC. To determine the relative copy-number of the *PIK3CA* gene, we employed real-time quantitative PCR to analyze 14 OSCC cell lines and 108 OSCC primary tumors. In each analysis, we used immortalized human oral keratinocytes RT7 as the normal counterpart of OSCC cell lines and two ESCC cell lines, which were previously described to harbor a 3q26.3 gain,[16–19] as a positive control. As shown in Fig. 2A, an increase in *PIK3CA* copy-number was detected in eight OSCC cell lines (57.1%, 1.2- to 2.6-fold amplification). In OSCC primary tumors, 18 specimens (16.7%, 1.3- to 3.4-fold amplification) showed an increase in copy-number (Table 3). However, the levels of *PIK3CA* amplification in these OSCC cell lines and primary tumors were much lower than those in the two ESCC cell lines (7.8- and 2.9-fold amplification, respectively).

Relationship between *PIK3CA* genetic alterations and clinical characteristics. We analyzed the correlation of *PIK3CA* mutations in exons 9 and 20 with clinical data of OSCC patients, and found a statistically significant association between the frequency of mutation and stage of disease ($P = 0.042$, Fisher's exact test)

Table 3. Clinical characteristics and *PIK3CA* genetic alterations in patients with oral squamous cell carcinoma

Patient no.	Age (years)	Sex	Ethnicity	Smoking status	Betel chewing	Tumor location	Histological grade	Stage	Mutation in exon 9 and 20	Copy number
1	75	M	Japanese	Former smoker	–	Buccal mucosa	Well	I	G1624A	Normal
2	58	F	Thai	Never-smoker	Yes	Gingiva	Moderate	I	Wild type	Normal
3	75	M	Thai	Never-smoker	No	Lip	Well	I	Wild type	Normal
4	70	F	Thai	Never-smoker	Yes	Tongue	Well	I	C3075T	Increased
5	34	F	Japanese	Current smoker	–	Tongue	Well	I	Wild type	Normal
6	36	F	Japanese	Never-smoker	–	Tongue	–	I	Wild type	Normal
7	38	M	Japanese	Current smoker	–	Tongue	Moderate	I	Wild type	Increased
8	42	F	Japanese	–	–	Tongue	–	I	Wild type	Increased
9	48	M	Japanese	–	–	Tongue	Well	I	Wild type	Normal
10	50	M	Japanese	Current smoker	–	Tongue	Moderate	I	Wild type	Normal
11	51	F	Japanese	Current smoker	–	Tongue	–	I	Wild type	Normal
12	53	M	Japanese	Never-smoker	–	Tongue	Moderate	I	Wild type	Normal
13	54	M	Japanese	Never-smoker	–	Tongue	–	I	Wild type	Normal
14	57	F	Japanese	Never-smoker	–	Tongue	Well	I	Wild type	Normal
15	59	M	Japanese	Current smoker	–	Tongue	–	I	Wild type	Normal
16	61	M	Japanese	Never-smoker	–	Tongue	Well	I	Wild type	Increased
17	61	M	Japanese	Current smoker	–	Tongue	Moderate	I	Wild type	Normal
18	64	M	Japanese	Current smoker	–	Tongue	Well	I	Wild type	Normal
19	65	F	Japanese	Never-smoker	–	Tongue	–	I	Wild type	Normal
20	65	M	Japanese	Current smoker	–	Tongue	–	I	Wild type	Normal
21	66	M	Japanese	Former smoker	–	Tongue	Moderate	I	Wild type	Normal
22	69	F	Japanese	–	–	Tongue	–	I	Wild type	Normal
23	69	M	Japanese	Never-smoker	–	Tongue	Moderate	I	Wild type	Normal
24	70	F	Japanese	–	–	Tongue	Well	I	Wild type	Normal
25	72	M	Japanese	Current smoker	–	Tongue	Moderate	I	Wild type	Normal
26	74	M	Japanese	–	–	Tongue	Well	I	Wild type	Normal
27	80	M	Japanese	–	–	Tongue	–	I	Wild type	Normal
28	80	M	Japanese	–	–	Tongue	–	I	Wild type	Normal
29	57	F	Thai	Never-smoker	No	Buccal mucosa	–	II	Wild type	Normal
30	81	F	Thai	Never-smoker	Yes	Buccal mucosa	Moderate	II	Wild type	Normal
31	85	F	Thai	Never-smoker	Yes	Buccal mucosa	Well	II	Wild type	Normal
32	52	F	Thai	Current smoker	–	Floor of mouth	–	II	Wild type	Normal
33	55	M	Thai	–	No	Floor of mouth	Moderate	II	A3127G	Increased
34	76	F	Thai	Never-smoker	No	Floor of mouth	–	II	Wild type	Normal
35	58	M	Thai	Current smoker	–	Gingiva	Well	II	Wild type	Normal
36	64	M	Thai	–	–	Gingiva	Well	II	Wild type	Increased
37	83	F	Thai	Never-smoker	Yes	Gingiva	Well	II	Wild type	Normal
38	68	F	Thai	Never-smoker	Yes	Lip	–	II	Wild type	Normal
39	79	F	Thai	Never-smoker	Yes	Lip	Moderate	II	Wild type	Normal
40	59	M	Thai	Never-smoker	No	Retromolar	Well	II	C3075T	Normal
41	28	M	Thai	Current smoker	No	Tongue	Well	II	Wild type	Normal
42	63	M	Thai	–	–	Tongue	Moderate	II	Wild type	Increased
43	71	M	Thai	Current smoker	No	Tongue	Well	II	C3075T	Normal
44	73	F	Thai	–	Yes	Tongue	Well	II	Wild type	Normal
45	74	M	Thai	Current smoker	–	Tongue	Well	II	Wild type	Increased
46	–	–	Japanese	–	–	Tongue	–	II	Wild type	Normal
47	–	–	Japanese	Current smoker	–	Tongue	–	II	Wild type	Normal
48	30	M	Japanese	Current smoker	–	Tongue	–	II	Wild type	Increased
49	41	M	Japanese	Current smoker	–	Tongue	–	II	Wild type	Increased
50	48	M	Japanese	Current smoker	–	Tongue	–	II	Wild type	Increased
51	49	M	Japanese	Never-smoker	–	Tongue	–	II	Wild type	Normal
52	50	M	Japanese	Current smoker	–	Tongue	Well	II	Wild type	Normal
53	50	F	Japanese	Current smoker	–	Tongue	Well	II	Wild type	Normal
54	56	M	Japanese	Never-smoker	–	Tongue	Well	II	Wild type	Normal
55	59	M	Japanese	–	–	Tongue	Well	II	Wild type	Normal
56	60	M	Japanese	Former smoker	–	Tongue	–	II	Wild type	Normal
57	61	M	Japanese	–	–	Tongue	–	II	G3145A	Normal
58	63	M	Japanese	Current smoker	–	Tongue	–	II	Wild type	Normal
59	63	M	Japanese	Former smoker	–	Tongue	–	II	Wild type	Normal
60	64	M	Japanese	–	–	Tongue	Well	II	Wild type	Normal
61	65	F	Japanese	Never-smoker	–	Tongue	–	II	Wild type	Normal
62	66	M	Japanese	Current smoker	–	Tongue	Moderate	II	Wild type	Normal
63	66	M	Japanese	–	–	Tongue	Well	II	Wild type	Normal
64	67	F	Japanese	Never-smoker	–	Tongue	–	II	Wild type	Normal
65	67	M	Japanese	Former smoker	–	Tongue	Moderate	II	Wild type	Normal
66	69	M	Japanese	Current smoker	–	Tongue	Well	II	Wild type	Normal
67	70	F	Japanese	Current smoker	–	Tongue	Well	II	Wild type	Normal

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Table 3. *continued*

Patient no.	Age (years)	Sex	Ethnicity	Smoking status	Betel chewing	Tumor location	Histological grade	Stage	Mutation in exon 9 and 20	Copy number
68	80	M	Japanese	Former smoker	–	Tongue	–	II	Wild type	Normal
69	51	M	Thai	–	–	Buccal mucosa	–	III	Wild type	Increased
70	75	F	Thai	–	Yes	Buccal mucosa	Well	III	Wild type	Normal
71	–	M	Thai	–	–	Floor of mouth	–	III	Wild type	Increased
72	79	F	Thai	Never-smoker	Yes	Gingiva	Well	III	Wild type	Normal
73	82	M	Thai	–	–	Gingiva	–	III	Wild type	Normal
74	72	M	Thai	Current smoker	–	Lip	–	III	Wild type	Normal
75	90	F	Thai	–	Yes	Tongue	Well	III	Wild type	Normal
76	–	–	Japanese	Current smoker	–	Tongue	Moderate	III	Wild type	Normal
77	44	F	Japanese	Never-smoker	–	Tongue	Well	III	Wild type	Normal
78	54	F	Thai	–	–	Buccal mucosa	–	IV	Wild type	Normal
79	59	F	Thai	–	Yes	Buccal mucosa	Well	IV	Wild type	Normal
80	64	M	Thai	Current smoker	Yes	Buccal mucosa	Well	IV	Wild type	Normal
81	65	M	Thai	Never-smoker	No	Buccal mucosa	Well	IV	Wild type	Normal
82	66	M	Thai	Current smoker	No	Buccal mucosa	Poor	IV	Wild type	Increased
83	67	F	Thai	–	–	Buccal mucosa	Well	IV	Wild type	Normal
84	69	M	Thai	Current smoker	–	Buccal mucosa	Well	IV	A1637T	Normal
85	74	F	Thai	Never-smoker	Yes	Buccal mucosa	Poor	IV	Wild type	Normal
86	75	M	Thai	Current smoker	–	Buccal mucosa	Well	IV	A3140G	Normal
87	77	F	Thai	Never-smoker	Yes	Buccal mucosa	Well	IV	G3145C	Normal
88	78	F	Thai	–	–	Buccal mucosa	Well	IV	Wild type	Normal
89	81	F	Thai	Never-smoker	Yes	Buccal mucosa	–	IV	Wild type	Normal
90	55	F	Thai	–	Yes	Floor of mouth	Well	IV	Wild type	Normal
91	55	M	Thai	Current smoker	–	Floor of mouth	Moderate	IV	Wild type	Normal
92	70	M	Thai	Never-smoker	No	Floor of mouth	Well	IV	Wild type	Normal
93	75	M	Thai	–	–	Floor of mouth	Poor	IV	Wild type	Normal
94	49	F	Thai	–	–	Gingiva	–	IV	Wild type	Normal
95	54	F	Thai	Never-smoker	No	Gingiva	Well	IV	Wild type	Normal
96	60	M	Thai	Current smoker	No	Gingiva	Well	IV	Wild type	Normal
97	61	F	Thai	Never-smoker	No	Gingiva	Well	IV	Wild type	Normal
98	66	M	Thai	Current smoker	No	Gingiva	Well	IV	Wild type	Increased
99	71	F	Thai	Current smoker	Yes	Gingiva	Moderate	IV	Wild type	Normal
100	79	F	Thai	Never-smoker	Yes	Gingiva	Well	IV	G1624A	Increased
101	82	F	Thai	–	Yes	Gingiva	Well	IV	C3075T	Normal
102	83	F	Thai	Never-smoker	Yes	Gingiva	Well	IV	Wild type	Increased
103	–	M	Thai	Current smoker	–	Gingiva	–	IV	G1633A	Increased
104	47	F	Thai	Current smoker	No	Hard palate	Well	IV	Wild type	Normal
105	40	F	Thai	Never-smoker	Yes	Tongue	Well	IV	Wild type	Normal
106	56	M	Thai	Current smoker	No	Tongue	Moderate	IV	Wild type	Normal
107	66	M	Thai	Current smoker	–	Tongue	Poor	IV	Wild type	Normal
108	72	F	Thai	Never-smoker	Yes	Tongue	Moderate	IV	Wild type	Normal
No. with genetic alterations (%)									8 (7.4%)	18 (16.7%)

(Table 4). The highest frequency of *PIK3CA* mutations was observed in stage IV of the disease (16.1%, 5/31), although all of the patients with stage IV disease were Thais (Table 3). There was, however, no significant difference between Japanese and Thais with regard to the frequency of *PIK3CA* mutations in a subset of patients with early stage (stage I–III) disease: 4% (2/50) versus 2% (1/27), respectively. In contrast, amplification of *PIK3CA* was detected in 14.3% (4/28), 17.5% (7/40), 22.2% (2/9), and 16.1% (5/31) of patients with stage I, II, III, and IV disease, respectively (Table 3). These frequencies were markedly lower than those reported for HNSCC,[11–13] and showed no association with disease stage ($P = 0.952$, chi-squared test). Therefore, our findings suggest that mutations in *PIK3CA* may be an oncogenic event at the advanced stages of OSCC, and that amplification of the gene may precede mutations in the development of OSCC.

There was no relationship between the presence of *PIK3CA* mutations and sex, ethnicity, smoking behavior, location, histological grading, or *PIK3CA* amplification (Table 4). In addition, there was no association between betel chewing and *PIK3CA* mutations in Thai patients ($P \geq 0.999$, Fisher's exact test).

We could not analyze the prognostic significance because the complete survival data were not included in our clinical data.

Effects of *PIK3CA* genetic alterations on *PIK3CA* expression and AKT activation in OSCC cell lines. To investigate which genetic event, mutation or amplification of *PIK3CA*, is crucial to the activation of the PI3K–AKT signaling pathway in OSCC, we analyzed the expression of *PIK3CA* and the phosphorylation of AKT in a panel of cell lines in which the *PIK3CA* and *PTEN* gene status was conformed. In the real-time RT-PCR analysis, we noted a weak tendency for high levels of *PIK3CA* mRNA in OSCC cell lines with *PIK3CA* amplification (Fig. 2A): five of eight OSCC cell lines with increased *PIK3CA* copy-numbers expressed the *PIK3CA* transcript at more than twofold the level found in RT7, while the expression level of *PIK3CA* in all of six OSCC cell lines with a normal *PIK3CA* copy-number changed less than twofold. However, the level of *PIK3CA* expression in the two ESCC cell lines was much higher than that in the OSCC cell lines.

Western blot analysis demonstrated that the phosphorylation of AKT at residues Ser-473 and Thr-308 was markedly increased

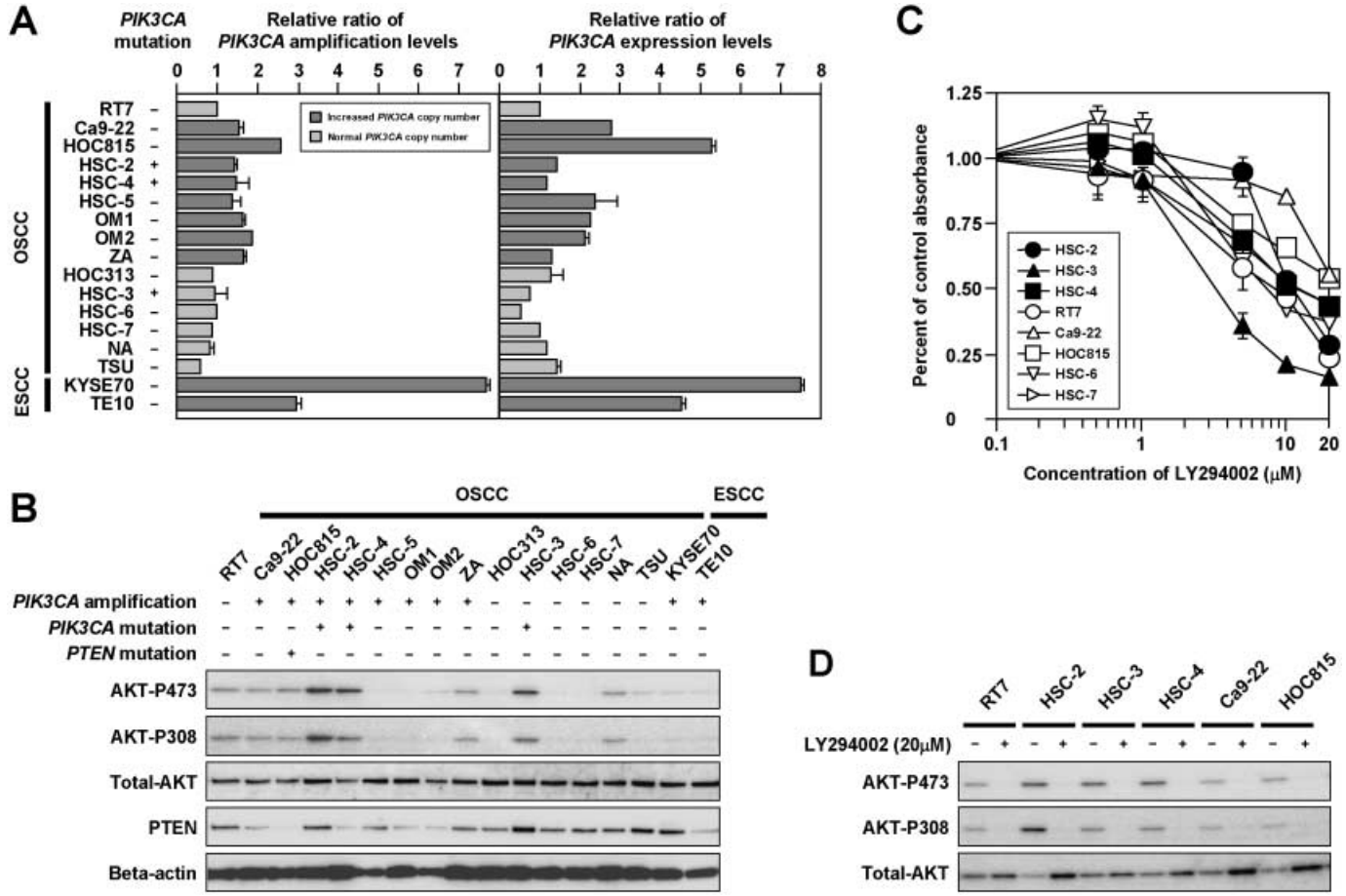

Fig. 2. Comparison of the effects of *PIK3CA* genetic alterations and LY294002 on the PI3K–AKT signaling pathway in a panel of OSCC cell lines. (A) Comparison of copy-number (*left*) and expression (*right*) of *PIK3CA* by real-time quantitative PCR. These results were normalized to the copy-number or expression levels of the *PIK3CA* gene in RT7 as a normal counterpart. (B) Comparison of AKT activation and PTEN protein expression levels by western blot analysis. To determine the increased phosphorylation of AKT, protein levels of total AKT in same samples were evaluated. Cell lines were cultured in 10% serum, and lysates prepared from them were immunoblotted with the indicated antibodies. (C) The effect of LY294002 on growth *in vitro*. Cell lines were cultured in 0.5% serum at the indicated concentration of LY294002 diluted in DMSO, and evaluated by the MTT method 7 days after treatments. These results were normalized to the cell numbers in control cultures treated with DMSO alone. (D) The effect of LY294002 on AKT activation. Cell lines were treated with 20 μM LY294002 or DMSO alone in medium containing 0.5% serum for 2 h, and evaluated by western blot analysis using the antibodies indicated.

in HSC-2, -3, and -4 with *PIK3CA* mutations (Fig. 2B). Ca9-22, HOC815, and ZA, which were high *PIK3CA* expressers with amplification in OSCC cell lines, also showed phosphorylated AKT. In addition, KYSE70 and TE10 were high expressers with *PIK3CA* amplification, whereas only a very weak phosphorylation of AKT was detected in both ESCC cell lines. The PTEN protein was sufficiently expressed in 13/14 of OSCC cell lines and RT7, but not in HOC815 harboring a *PTEN* mutation. Notably, no clear correlation was found between protein expression levels of PTEN and the activation of AKT in these cell lines. Phosphorylation of AKT detected in RT7 was thought to have been increased by recombinant EGF added in the culture medium. Although NA without a genetic alteration of *PIK3CA* or *PTEN* also showed phosphorylated AKT, it was unknown why the phosphorylation increased in this cell line. Hence, these results indicate that the mutation, rather than an increase in the copy-number, of *PIK3CA* may play a crucial role in the activation of the PI3K–AKT signaling pathway.

Effects of LY294002 on cell growth and AKT activation in OSCC cell lines. Next, we compared the effects of LY294002, a commonly used PI3K inhibitor, on RT7 and OSCC cell lines with and without mutant forms of PIK3CA *in vitro*. LY294002 was shown to inhibit cell growth in a dose-dependent manner. However, no difference in the inhibitory effects of LY294002 on *in vitro* growth rates was seen among cell lines analyzed, regardless of the mutation, expression, or amplification of *PIK3CA* (Fig. 2C). LY294002 inhibited the phosphorylation of AKT in all cell lines analyzed (Fig. 2D). Thus, the inhibitory effects of LY294002 on cell growth were not related to the existence of *PIK3CA* genetic abnormalities.

Discussion

The PI3K–AKT pathway, as well as Ras and p53, are important signaling pathways contributing to cancer development, and are aberrantly activated in many types of cancers by gain- or loss-of-function of components of this pathway, such as *PTEN*, *AKT* and *PIK3CA*.[1–3] Here we performed a large-scale mutational analysis of cell lines and primary tumors of OSCC, and found a significant correlation between the advanced stage of OSCC and the frequency with which *PIK3CA* is mutated in exons 9 and 20 ($P = 0.042$). The present study is the first report to mention a significant correlation between *PIK3CA* mutations and disease stage in OSCC. The frequency of mutations of the *PIK3CA* gene

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Table 4. Correlations between *PIK3CA* mutations and clinicopathological characteristics of patients with oral squamous cell carcinoma

Characteristic	*PIK3CA* mutation	No *PIK3CA* mutation	*P*-value
Total number (%)	8 (7.4%)	100 (92.6%)	
Age (years) [median (range)]	75 (55–79)	64 (28–90)	
Sex			
Male	6	54	$P = 0.462$*
Female	2	43	
Unknown	0	3	
Ethnicity			
Japanese	2	48	$P = 0.282$*
Thai	6	52	
Smoking status			
Ever-smokers	4	41	$P = 0.691$*
Never-smokers	2	33	
Unknown	2	26	
Location			
Buccal mucosa	4	14	$P = 0.123$†
Floor of mouth	1	7	
Gingiva	2	14	
Hard palate	0	1	
Lip	0	4	
Retromolar	0	1	
Tongue	1	59	
Histological grading			
Well	5	45	$P = 0.674$†
Moderate	1	18	
Poor	0	4	
Unknown	2	33	
Disease stage			
I–III	3	74	$P = 0.042$*
IV	5	26	
PIK3CA amplification			
Positive	3	15	$P = 0.127$*
Negative	5	85	

*Fisher's exact test; †chi-squared test.

has been reported to be 32% in colon cancer, 3–27% in brain tumors, 4–25% in gastric cancer, 8–40% in breast cancer, 4% in lung cancer, 4–7% in ovarian cancer, and 11% in HNSCC.[4,15,20–23] In our study, the frequency of mutations in cell lines (21.4%) and primary tumors (7.4%) of OSCC was not as high as in previous studies, although *PIK3CA* was mutated at a relatively high frequency in stage IV (16.1%), suggesting that *PIK3CA* mutations may be a late event in genomic aberration involved in the progression of OSCC. The frequency of *PIK3CA* mutations in OSCC specimens could be even lower than that observed in OSCC cell lines, as activation of the PI3K–AKT pathway may have conferred a selective growth advantage *in vitro*,[6–9] leading to the successful establishment of cell lines. However, it has also been reported that *PIK3CA* mutations were detected in the early lesions of various cancers, such as intraductal carcinoma of breast cancer (26.9%), dysplastic nodule of hepatocellular carcinoma (35.6%), and early gastric cancer (6.5%).[24] Taken together, those results suggest that the clinicopathological significance of *PIK3CA* mutations may differ according to the type of cancer.

Amplification of the *PIK3CA* gene has been reported in various human cancers,[1,25] and described as a genomic aberration in the early stages of HNSCC.[11,12] Consistent with these reports, our study showed that the copy-number of *PIK3CA* was also increased in the early stages of OSCC, independent of the stage of the disease ($P = 0.952$). In addition, the phosphorylation of AKT was considerably increased in OSCC cell lines with *PIK3CA* mutations compared with those without mutations, despite the amplification. Recently, Samuels *et al*. clearly showed that overexpression of a hotspot mutant *PIK3CA* remarkably increased the phosphorylation of AKT in comparison to overexpression of wild-type *PIK3CA in vitro*.[9] In their study, the increased AKT activation was due to increased PIK3CA kinase activity in the mutant form, not increased protein levels of *PIK3CA*, indirectly supporting our findings *in vitro*. Another point, which should be mentioned, was that the levels of increase in the copy-number of *PIK3CA* were very low in cell lines and primary tumors of OSCC as compared with ESCC cell lines.[16–19] Therefore, the activation of *PIK3CA* due to mutation, rather than amplification, may strongly contribute to the dysregulation of the PI3K–AKT signaling pathway in the advanced progression, especially stage IV, of OSCC.

One of the most impressive recent developments in cancer therapy has been the use of kinase inhibitors such as imanitib (Gleevec), trastzumab (Herceptin), and gefitinib (Iressa).[26–28] Sensitivity to gefitinib, an EGFR-specific tyrosine kinase inhibitor, was reported to be significantly higher in non-small-cell lung cancer patients with *EGFR* mutations than those without these mutations.[29,30] A mutation in the kinase domain of *EGFR* was reported to be rare in HNSCC (1–7%),[31,32] and was not detected in OSCC cell lines examined in this study. Thus, we look forward to development of molecules, other than EGFR, as therapeutic targets in OSCC. PIK3CA could be a potential target for cancer therapy, although its regulated signaling pathway is very important even in normal cells. LY294002, a commonly used pharmacological inhibitor of the cAMP-independent PI3K–AKT signal transduction pathway, has been shown to inhibit the growth of tumors with an activated PI3K–AKT pathway *in vitro* and *in vivo*.[9,33] However, our *in vitro* study demonstrated that LY294002

inhibited cell growth and AKT activation in tumor cells regardless of the presence of *PIK3CA* mutations. These *in vitro* data strongly suggest that the development of *PIK3CA* mutation-specific inhibitors is important for cancer therapy in the clinical setting.

In summary, our findings suggest that: (i) *PIK3CA* mutations may be an oncogenic aberration at advanced stages of OSCC; (ii) the mutation, rather than increased DNA copy-number, of *PIK3CA* is likely to function as an oncogene in OSCC; and (iii) as PIK3CA could be a promising target for cancer therapies, further development of *PIK3CA* mutation-specific inhibitors is warranted because of the central importance of the PI3K–AKT nexus in normal and tumor cells.

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References

1 Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002; **2**: 489–501.
2 Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. *Nat Med* 2004; **10**: 789–99.
3 Cully M, You H, Levine AJ, Mak TW. Beyond *PTEN* mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. *Nat Rev Cancer* 2006; **6**: 184–92.
4 Samuels Y, Wang Z, Bardelli A *et al*. High frequency of mutations of the *PIK3CA* gene in human cancers. *Science* 2004; **304**: 554.
5 Bader AG, Kang S, Zhao L, Vogt PK. Oncogenic PI3K deregulates transcription and translation. *Nat Rev Cancer* 2005; **5**: 921–9.
6 Ikenoue T, Kanai F, Hikiba Y *et al*. Functional analysis of *PIK3CA* gene mutations in human colorectal cancer. *Cancer Res* 2005; **65**: 4562–7.
7 Isakoff SJ, Engelman JA, Irie HY *et al*. Breast cancer-associated *PIK3CA* mutations are oncogenic in mammary epithelial cells. *Cancer Res* 2005; **65**: 10992–1000.
8 Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. *Proc Natl Acad Sci USA* 2005; **102**: 802–7.
9 Samuels Y, Diaz LA Jr, Schmidt-Kittler O *et al*. Mutant *PIK3CA* promotes cell growth and invasion of human cancer cells. *Cancer Cell* 2005; **7**: 561–73.
10 Nomura K, Sobue T, Nakatani H *et al*. Number of deaths and proportional mortality rates from malignant neoplasms by site in Japan (2003). In: Editorial Board of Cancer Statistics in Japan, eds. *Cancer Statistics in Japan 2005*. Tokyo: National Cancer Center, 2005; 36–9. Available from: http://www.ncc.go.jp/en/statistics/index.html
11 Redon R, Muller D, Caulee K, Wanherdrick K, Abecassis J, du Manoir S. A simple specific pattern of chromosomal aberrations at early stages of head and neck squamous cell carcinomas: *PIK3CA* but not *p63* gene as a likely target of 3q26-qter gains. *Cancer Res* 2001; **61**: 4122–9.
12 Woenckhaus J, Steger K, Werner E *et al*. Genomic gain of PIK3CA and increased expression of p110alpha are associated with progression of dysplasia into invasive squamous cell carcinoma. *J Pathol* 2002; **198**: 335–42.
13 Pedrero JM, Carracedo DG, Pinto CM *et al*. Frequent genetic and biochemical alterations of the PI 3-K/AKT/PTEN pathway in head and neck squamous cell carcinoma. *Int J Cancer* 2005; **114**: 242–8.
14 Lin M, Smith LT, Smiraglia DJ, Kazhiyur-Mannar R *et al*. DNA copy number gains in head and neck squamous cell carcinoma. *Oncogene* 2006; **25**: 1424–33.
15 Qiu W, Schonleben F, Li X *et al*. *PIK3CA* mutations in head and neck squamous cell carcinoma. *Clin Cancer Res* 2006; **12**: 1441–6.
16 Shinomiya T, Mori T, Ariyama Y *et al*. Comparative genomic hybridization of squamous cell carcinoma of the esophagus: the possible involvement of the DPI gene in the 13q34 amplicon. *Genes Chromosomes Cancer* 1999; **24**: 337–44.
17 Imoto I, Pimkhaokham A, Fukuda Y *et al*. SNO is a probable target for gene amplification at 3q26 in squamous-cell carcinomas of the esophagus. *Biochem Biophys Res Commun* 2001; **286**: 559–65.
18 Imoto I, Yuki Y, Sonoda I *et al*. Identification of ZASC1 encoding a Kruppel-like zinc finger protein as a novel target for 3q26 amplification in esophageal squamous cell carcinomas. *Cancer Res* 2003; **63**: 5691–6.
19 Sonoda I, Imoto I, Inoue J *et al*. Frequent silencing of low density lipoprotein receptor-related protein 1B (LRP1B) expression by genetic and epigenetic mechanisms in esophageal squamous cell carcinoma. *Cancer Res* 2004; **64**: 3741–7.
20 Broderick DK, Di C, Parrett TJ *et al*. Mutations of *PIK3CA* in anaplastic oligodendrogliomas, high-grade astrocytomas, and medulloblastomas. *Cancer Res* 2004; **64**: 5048–50.
21 Campbell IG, Russell SE, Choong DY *et al*. Mutation of the *PIK3CA* gene in ovarian and breast cancer. *Cancer Res* 2004; **64**: 7678–81.
22 Li VS, Wong CW, Chan TL *et al*. Mutations of *PIK3CA* in gastric adenocarcinoma. *BMC Cancer* 2005; **5**: 29.
23 Wang Y, Helland A, Holm R, Kristensen GB, Borresen-Dale AL. *PIK3CA* mutations in advanced ovarian carcinomas. *Hum Mutat* 2005; **25**: 322.
24 Lee JW, Soung YH, Kim SY *et al*. *PIK3CA* gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene* 2005; **24**: 1477–80.
25 Shayesteh L, Lu Y, Kuo WL *et al*. *PIK3CA* is implicated as an oncogene in ovarian cancer. *Nat Genet* 1999; **21**: 99–102.
26 Druker BJ, Talpaz M, Resta DJ *et al*. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001; **344**: 1031–7.
27 Couzin J. Cancer drugs. Smart weapons prove tough to design. *Science* 2002; **298**: 522–5.
28 Green MR. Targeting targeted therapy. *N Engl J Med* 2004; **350**: 2191–3.
29 Dowell JE, Minna JD. The impact of epidermal-growth-factor-receptor mutations in response to lung-cancer therapy. *Nat Clin Pract Oncol* 2004; **1**: 2–3.
30 Paez JG, Janne PA, Lee JC *et al*. *EGFR* mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 2004; **304**: 1497–500.
31 Lee JW, Soung YH, Kim SY *et al*. Somatic mutations of *EGFR* gene in squamous cell carcinoma of the head and neck. *Clin Cancer Res* 2005; **11**: 2879–82.
32 Loeffler-Ragg J, Witsch-Baumgartner M, Tzankov A *et al*. Low incidence of mutations in *EGFR* kinase domain in Caucasian patients with head and neck squamous cell carcinoma. *Eur J Cancer* 2006; **42**: 109–11.
33 Hu L, Zaloudek C, Mills GB, Gray J, Jaffe RB. *In vivo* and *in vitro* ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin Cancer Res* 2000; **6**: 880–6.

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